The pro-apoptotic action of the peptide hormone, *Neb*-colloostatin, on insect haemocytes

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SUMMARY

The gonadoinhibitory peptide hormone, *Neb*-colloostatin, was first isolated from ovaries of the flesh fly *Neobellieria bullata*. This 19-mer peptide is thought to be a cleaved product of a collagen-like precursor molecule that is formed during remodelling of the extracellular matrix. In this study, we report that upon injection of pico- and nanomolar doses, this peptide exerts a pro-apoptotic action on haemocytes of *Tenebrio molitor* adults, as visualized by changes in morphology and viability. The F-actin cytoskeleton was found aggregating into distinctive patches. This may be responsible for the observed inhibition of adhesion of haemocytes and for the stimulation of filopodia formation. However, *Neb*-colloostatin injection did not induce the formation of authophagic vacuoles. Our results suggest that physiological concentrations of *Neb*-colloostatin may play an important role in controlling the quantity and activity of haemocytes in insect haemolymph. They also suggest that in periods that *Neb*-colloostatin is released, this peptide may cause a weakening of the insects' immune system. This is the first report that exposure to a peptide hormone causes apoptosis in insect haemocytes.

INTRODUCTION

Insect haemocytes engage in immune responses that integrate humoral and cellular components to eliminate pathogens. Cellular immune reactions can be modulated by various endogenous factors such as: eicosanoides (Franssens et al., 2005), biogenic amines (Baines et al., 1992), 20-hydroxyecdysone and juvenile hormone (Franssens et al., 2006). Injection of ecdysone into mid- third-instar *Drosophila melanogaster* larvae made plasmatocytes differentiate into actively phagocyting macrophages. This hormone was also shown to be necessary for post-embryonic haematopoiesis and to stimulate the encapsulation of parasites (Lanot et al., 2001; Sorrentino et al., 2002). In contrast, injection of juvenile hormone suppressed the encapsulation response in *Tenebrio molitor* (Rantala et al., 2003). The *in vivo* effects of some peptide hormones on nodule formation and on the activation of the prophenoloxidase cascade in insect haemolymph have also been studied. In *Locusta migratoria*, Goldsworthy et al. (2002; 2003a; 2003b) showed that co-injection of adipokinetic hormone-I (*Lom*-AKH-I) with immunogens such as the bacterial lipopolysaccharide (LPS) or β-1,3-glucan (laminarin) stimulated nodule formation in larval and adult locusts. It also activated prophenoloxidase activity in the haemolymph of sexually mature adults. Eicosanoid synthesis is important for nodule formation, but not for increasing the phenoloxidase activity. In addition, Skiner et al. (1997) detected allatostatin-like immunoreactive material in

haemocytes of the cockroach *Diploptera punctata.* Franchini et al. (1996) reported the presence of adrenocorticotropin hormone (ACTH)-like molecules in haemocytes of newly enclosed *Calliphora vomitoria* adults. The presence of these molecules in haemocytes suggested some as yet unknown regulatory role. Altogether, there is some evidence for the interaction between the endocrine and the immune systems, but it is far from complete. Further analysis is required to yield more insight into the involvement of the endocrine system in the regulation of haemocyte populations and cellular immunity.

In a screen for other biologically active peptide hormones, the influence of *Neb*-colloostatin, a gonadoinhibitory peptide with pleiotropic activity in insects, on haemocytes has been studied. *Neb*-colloostatin is, unlike most insect peptide hormones, not a neuropeptide. It is thought to be a cleaved product of a collagen-like precursor molecule that is formed during remodelling of the extracellular matrix (De Loof et al., 1995). Upon injection, it inhibits ovarian development in the flesh fly *N. bullata* (Bylemans et al., 1995) and in the mealworm *T. molitor* (Kuczer et al., 2007). It inhibits oocyte growth, it reduces the number of eggs and their hatchability, and it delays the embryonic development in *T. molitor* (Wasielewski and Rosiński, 2007). Bylemans et al. (1995) showed that *Neb*-colloostatin inhibits vitellogenin biosynthesis, but that this effect is not mediated by inhibiting trypsin- or ecdysone biosynthesis.

In this study, a new physiological effect of *Neb*-colloostatin in insects was detected, namely a pro-apoptotic action on haemocytes of *T. molitor* adults.

MATERIALS AND METHODS

Insects

A stock culture of *T. molitor* was maintained at the Department of Animal Physiology and Development as described previously (Rosiński et al., 1979). Studies were carried out on 4-days old adult beetles. As the mealworm parents' age is important for the developmental features of their offspring (Ludwig and Fiore, 1960; Ludwig et al., 1962; Rosinski, 1995), all insects in our experiments derived from less then 1 month old parents.

Peptide synthesis

Neb-colloostatin was synthesized by the classical solid phase method based on the Fmoc-protocol (Fields and Nobel, 1990) as described previously (Kuczer et al., 2004). Briefly, amino acids were assembled on a 9-fluorenylmethoxycarbonyl-Arg(2,2,5,7,8-pentamethylchromane-6-sulfonyl)-Wang resin. 2-(1H-benzotriazole-1-yl)-1,1,3,3,-

tetramethyluronium hexafluorophosphate (HBTU) in the presence of 1-hydroxybenzotriazole (HOBt) and *N*-ethylmorpholine (NEM) were used as coupling reagents. The *N*-Fmoc group was removed with 20% piperidine in *N*, *N*-dimethylformamide (DMF). The peptide-resin was cleaved with trifluoroacetic acid (TFA) in the presence of ethanedithiol (EDT). The peptide was purified by preparative high performance liquid chromatography on a Varian ProStar, column: Tosoh Biosciences ODS-120T C18 (ODS 300 × 21.5 mm). Analytical HPLC was performed on a Thermo Separation Products HPLC system with a Vydac C18 column (ODS 250×4.6 mm) with a linear gradient 0–100% B in A (A - 0.1% aqueous TFA, B - 80% acetonitrile in water, containing 0.1% TFA) for 60 min at a flow rate of 1 ml/min with UV absorption determined at 210 nm. The molecular weight of the peptide was determined with a Bruker Daltonics microTOF-Q mass spectrometer. The final product purity was checked by HPLC, TLC, optical activity and molecular weight determinations. Analytical HPLC was performed on a Thermo Separation Products HPLC system with a Vydac C18 column (ODS 250 4.6 mm) with a linear gradient 0–100% B in A for 60 min at a flow rate of 1 ml/min with the UV absorption determined at 210 nm. The optical activity of the chiral compound was measured with a Jasco DIP-1000 polarimeter (Jasco). TLC was performed on the aluminum sheets precoated with silica gel 60 from Merck.

The peptide was dissolved in physiological saline for *Tenebrio* (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$) (Rosiński, 1995) to yield a stock solution of 1 mM, and it was stored at -30 ºC. The working dilutions from the stock solution were made in saline.

Injection procedure, haemolymph collection

The beetles were anaesthetised with CO_2, washed in distilled water and disinfected with 70% ethanol. *Neb*-colloostatin was injected (2 µl, in dose of 1 or 10 nmole of *Neb*-colloostatin per insect) through the ventral membrane between the second and the third abdominal segments towards the head with a Hamilton syringe (Hamilton Co.). The control insects were injected with the same volume of physiological saline. All solutions were sterilised through the 0.22 µm pore filter membrane (Millipore) and all injections were performed in sterile conditions.

Before haemolymph collection, the beetles were anaesthetised again with CO_2, washed in distilled water and disinfected with 70% ethanol. Both control and injected insects were taken 1 hour after injection, and the haemocytes were prepared for the living- as well as for the fixed conditions. Upon cutting off a tarsus from a foreleg, haemolymph samples (5 µl), were collected with “end to end” microcapillaries (Drummond). They were diluted in 20 µl of

ice-cold physiological saline containing anticoagulant buffer (4.5 mM citric acid and 9 mM sodium citrate) in a 5:1 v/v ratio.

Haemolymph from control- and from peptide-injected insects was dropped on alcohol-cleaned cover glasses coated with 7 µl 0.01% poly-L-lysine (Sigma P4707). After allowing haemocytes to settle (15 min, at room temperature), the remaining fluid was removed and the remaining cells were washed twice with physiological saline. Fixation was achieved in 4% paraformaldehyde. The prepared haemocytes were used for the appropriate microscopic analysis (mitochondrial staining, activation of caspases, F-actin microfilament staining and detection of autophagic vacuoles).

The *Neb*-colloostatin *in vitro* haemocyte' assay

Control haemocytes were incubated in physiological saline, whereas the experimental haemocytes were incubated in 1 nM or 10 nM *Neb*-colloostatin solutions for 1h at RT. Next, haemocytes were fixed in 4% paraformaldehyde for 10 min at room temperature, and washed twice with physiological solution. The haemocytes were stained for activated caspase activity as described below, and the percentage of apoptosing cells was scored. In addition, the *in vitro* peptide-treated haemocytes were stained with mono-dansyl-cadaverin (MDC) to show the autophagic vacuoles).

Assay for active mitochondria

To label mitochondria, both the control- and the haemocytes of insects injected with peptide were incubated with 700 nM MitoTracker®Green FM (Invitrogen) in physiological solution for 30 min at room temperature in the dark. After staining, the haemocytes were washed twice with the physiological saline and they were immediately examined with a Nikon Eclipse TE 2000-U fluorescence microscope. The images were made with a Nikon DS-1QM digital camera.

***In situ* assay for activated caspases, detection of F-actin microfilaments and counterstaining of the nucleus**

The presence of active caspases was searched for by using a sulphorhodamine derivative of valylalanylaspartic acid fluoromethyl ketone, a potent inhibitor of caspase activity (SR-VAD-FMK) (in accordance to the manufacturer's instructions of the sulphorhodamine multi-caspase activity kit, AK-115, BIOMOL, PA). Haemolymph samples from control- and from 4-day old insects injected with *Neb*-colloostatin were dropped on poly-L-lysine-coated cover

slips (18 x 18 mm) and haemocytes were allowed to adhere for 15 min. Next, they were rinsed with physiological saline, incubated in reaction medium (1/3x SR-VAD-FMK) for 1 h at room temperature in the dark, rinsed again three times with wash buffer for 5 min at room temperature and finally fixed in 3.7% paraformaldehyde for 10 min. The prepared haemocytes were studied with a Zeiss LSM 510 confocal laser scanning microscope with filters set for rhodamine (excitation 543 nm and emission 560 nm) and the percentage of the apoptosing cells was scored.

For visualizing F-actin microfilaments, haemocytes that had first been stained for caspase activity as described above were permeabilized in 3.7% paraformaldehyde in physiological saline containing 0.1% Triton X-100 for 5 min at room temperature. Next, the haemocytes were washed in physiological saline and stained with Oregon Green® 488 phalloidin (Invitrogen) for 20 min at room temperature in the dark, in accordance to the manufacturer's instructions. After washing again in physiological saline, the haemocytes were stained with freshly prepared solutions of Hoechst-33258 (Invitrogen) in physiological saline. Incubation in the dark lasted for 5 min. Thereafter, the haemocytes were washed once with distilled water, they were mounted using a mounting medium, and examined with a Nikon Eclipse TE 2000-U fluorescence microscope.

Mono-dansyl-cadaverine (MDC) staining for autophagic vacuoles

Autophagic vacuoles were searched for with the mono-dansyl-cadaverin (MDC) staining, according to the method of Biederbick et al. (1995). Cover slips with adhering haemocytes were washed with physiological saline and incubated with 0.05 mM MDC for 15 min at room temperature in the dark. Next, haemocytes were washed three times with physiological saline. The percentage of MDC-positive cells was performed immediately after preparation using a Nikon Eclipse TE 2000-U fluorescence microscope.

RESULTS

Our first marker of proapoptotic activity was activation of caspases. One hour after injection of *Neb*-colloostatin injection in a dose of 1 or 10 nmole per insect, caspase activity (1-9) has been detected (Fig. 1B, C, E, F). The degree of activation depended on the applied hormone dose. In all studied individuals, injection of 10 nmole of peptide caused the activation of caspases in all haemocytes (Fig. 1F), whereas in insects injected with 1 nmole of *Neb*-colloostatin about 67% of haemocytes displayed caspase activity (Fig. 1E). Moreover,

exposure to *Neb*-colloostatin induced in the haemocytes malformations that are typical for an apoptotic morphology, such as cell shrinkage, rounding up and extensive membrane blebbing (Fig. 1F). The *in vitro* bioassay on haemocytes glued to cover slips confirmed the pro-apoptotic activity of *Neb*-colloostatin. The 1 nM solution of this peptide was very potent: it activated caspases in 100% of the haemocytes (Fig. 2).

The second marker concerned changes in mitochondrial membrane potential. Our fluorescence microscopy analysis revealed that *Neb*-colloostatin injections resulted in the loss of mitochondrial membrane potential as shown in Fig. 1H.

As to our third marker, namely rearrangements in the cytoskeleton, these could be visualized upon staining the actin cytoskeletal network distribution and arrangement with Oregon Green® 488 phalloidin. As shown in Fig. 1A, control haemocytes formed extensive filopodia, in which the F-actin microfilaments were detected after the cells had been allowed to adhere to the cover slips for 15 min. Injection of *Neb*-colloostatin in a dose of 1 nmole per individual resulted in the accumulation of large F-actin aggregates (Fig. 1B) and in the formation of membrane blebs (Fig. 1C). In control haemocytes no membrane blebs have been observed. In the *in vivo* experiment, changes in F-actin distribution after 1 hour treatment with *Neb*-colloostatin were so pronounced that the cells' morphology was drastically changed. Moreover, the peptide-stimulated haemocytes were less able to form pseudopodia in comparison to the control haemocytes.

In order to examine to which type of programmed cell death, type I or type II (see discussion), injection of *Neb*-colloostatin was causal, the MDC staining method was used for tracing the induction of autophagic vacuoles. The results in both the *in vivo* injection- and *in vitro* incubation experiments were negative as no autophagic vacuoles at all could be visualized (data not shown).

DISCUSSION

The novelty of this study is showing for the first time pro-apoptotic activity of *Neb*-colloostatin on the insect haemocytes.

Cells can respond to stress stimuli in a variety of ways ranging from activation of pathways that promote survival to eliciting programmed cell death or even the execution of necrotic cell death that eliminates damaged cells (Fulda et al., 2010). Programmed cell death can be classified into two major groups according to its morphological features: (1) type I of programmed cell death - apoptosis and (2) type II of programmed cell death - autophagic cell

death. Apoptosis can result from a genetic program regulating the cell number in physiological and pathological conditions. It can also be induced in response to environmental changes or exposures to the variety of stresses and cytotoxic drugs (Lee et al., 1997; Verheij et al., 1996; Zanke et al., 1996). Apoptosis is characterized by mitochondrial dysfunction, nuclear and cytoplasmic condensation, preservation of organelles from autophagic degradation, cell fragmentation into apoptotic bodies and removal of such bodies via phagocytosis. This process involves the activation of a family of cysteine proteases, caspases, which recognise and cleave cellular target proteins leading to cell death (Nicholson, 1999).

In the *in vivo* test, within 1 h after injection *Neb*-colloostatin (10 nmole peptide/insect) induced caspase activation as well as marked morphological changes. The haemocytes' ability to form filopodia during adhesion decreased significantly and, as a consequence, most haemocytes became round. *T. molitor* haemocytes start producing numerous circular blebs, a marker for disintegration, but they do not display autophagic activity. This haemocyte phenotype is similar to that observed in cell types undergoing type I of programmed cell death-apoptosis. Terahara et al. (2003), using *in vitro* experiments, described similar changes in the morphology of haemocytes of the Pacific oyster, *Crassostrea gigas*. The morphology was affected both Arg-Gly-Asp and Arg-Gly-Glu peptides.

Mitochondrial degeneration is another characteristic effect of *Neb*-colloostatin on *T. molitor* haemocytes. Mitochondria are involved in induction of apoptosis *via* caspase activation and cytochrome c release (Desagher and Martinou, 2000; Kluck et al., 1997). In some apoptotic systems, the loss of membrane polarization may be an early event in the apoptotic process that leads the release of apoptogenic factors and to loss of oxidative phosphorylation (Düßmann et al., 2003). In *T. molitor* haemocytes, *Neb*-colloostatin injection caused swelling of the mitochondria and loss of their membrane potential suggesting a permeabilizing effect on mitochondrial membranes. According to Gourlay et al. (2004), rearrangements of the F-actin cytoskeleton may be causal to such effects. It is known, that the cytoskeleton is responsible for mitochondrial movement within cells and for mitochondrial membrane potential maintenance. In insects injected with *Neb*-colloostatin, F-actin staining showed cytoskeletal disintegration, as indicated by the appearance of a number of intensively stained foci. The observed F-actin staining pattern is similar to the one achieved by treating rock oyster (*Saccostrea glomerata*) haemocytes with noradrenaline (Aladaileh et al., 2008). MAP-dependent F-actin rearrangement mediates membrane blebbing during stress-induced apoptosis contributing to cell death (Huot et al., 1998). Taken together, the increased caspase activity, the rearrangement of the F-actin cytoskeleton, the blebbing phenotype and the

disintegration of the hemocytes are indicative for a proapoptotic effect of *Neb*-colloostation on haemocytes of *T. molitor*. Apoptosis is often linked to stress stimuli (for review see Gores et al., 1990; Mills et al., 1998).

A study performed earlier by Wasielewski and Rosiński (2007) showed that injection of approximately 2 mmole of *Neb*-coollostatin into *T. molitor* females on days 1, 2, 3 of the first reproductive cycle strongly inhibited ovarian growth and oocyte development. It should be noted that the peptide doses used in this work were significantly lower. The approximate blood volume of adult *T. molitor* blood amounts to 20 μl. This means that the *Neb*-coollostatin concentrations in the haemolymph of the injected mealworms reached picomolar concentrations (50 or 500, respectively). This calculation shows that the studied peptide is very potent and that the effects we observed can be induced at physiological concentrations. As a consequence the effects are not pharmacological artefacts.

In general, the insect defence system against invading parasites and pathogens involves both cellular and humoral immune responses. To fight infection, haemocytes phagocytose, form nodules or encapsulate foreign invaders and they produce humoral defence molecules (for a review see: Lavine and Strand, 2002). Therefore, maintaining a correct number of healthy haemocytes is crucial for the insects' survival. The present study is the first to indicate that synthetic pro-apoptotic peptide *Neb*-colloostatin exerts a potent proapoptotic effect on *T. molitor* haemocytes that could disrupt their immunological functions. Moreover, the decreased ability of *Neb*-colloostatin-treated haemocytes to adhere and to form filopodia suggests impairment of adhesion of circulating haemocytes to sites of injury or infection.

In the literature, only few studies evidenced an immunomodulatory role of some peptide hormones. For example, it was found that plasmatocytes and granular cells of *Calliphora vomitoria* show immunoreactive ACTH and TNF-α-like molecules when they are activated and recruited into capsule formation, whereas the freely circulating plasmatocytes, are not involved in encapsuluation and do not express these molecules. Moreover, ACTH-like molecules are also permanently present in phagocyting haemocytes (Franchini et al. 1996). These authors suggested that ACTH-like molecules play a physiological role in capsule formation and could act as a chemoattractant to other haemocytes.

In cockroaches and crickets, inhibition of juvenile hormone biosynthesis by the corpora allata is the best documented function of allatostatins (Woodhead et al., 1989; 1993; Lorenz et al., 1995). These neuropeptides were isolated for the first time from the brain extracts of the cockroach *Diploptera punctata* (Pratt et al., 1989; Woodhead et al., 1989). However, Skinner et al. (1997) demonstrated that allatostatins are also present in and synthesized by cockroach

granular haemocytes. In addition, Garside et al. (1997) showed that allatostatins are rapidly degraded in haemolymph. According to Hoffmann et al. (1999) these findings could suggest that allatostatin-containing haemocytes are acting locally e.g. to regulate specific functions of other haemocytes, but to this day, the action of these peptides on cellular and humoral immune responses in insects remains to be elucidated.

Goldsworthy et al. (2002, 2003) studied *in vivo* interactions between the locust endocrine and immune systems in relation to nodule formation and the activation of the prophenoloxidase cascade in haemolymph. They showed, for the first time, the immunostimulatory effects of co-injection of adipokinetic hormone-I (*Lom*-AKH-I, AKH) with microbial cells components such as β-1,3 glucan or bacterial lipopolysaccharide (LPS) in haemolymph of *Locusta migratoria*. Such co-injection prolonged or facilitated phenoloxidase activation in haemolymph of adult locust. It also increased nodule formation in a dose-dependent manner and in a very defined pattern reflecting the distribution of reticular cells. Goldsworthy et al. (2003) also suggest that in *L. migratoria* the recruitment of haemocytes from haemopoietic tissue occurs in response to injections of LPS. Otherwise, the number of haemocytes in haemolymph would drop dramatically and it would severely limit further nodule formation.

In conclusion, our study shows for the first time that *Neb*-colloostatin is a peptide hormone that exerts pro-apoptotic activity on insect haemocytes. Injection of *Neb*-colloostatin in physiological concentrations results in significant hemocytotoxicity and a marked increase in apoptotic activity in haemocytes. Apoptosis induced by *Neb*-colloostatin may have important implications for the insect's immune defence, resulting in weakening of the immune system due to loss of the haemocyte activity. However, the molecular mechanism underlying the activation of the apoptotic programme in haemocytes by *Neb*-colloostatin remains unknown and requires further studies. On the other hand, the obtained results suggest the physiological concentrations of *Neb*-colloostatin could play an important role in the control of haemocytic activity in haemolymph of insects.

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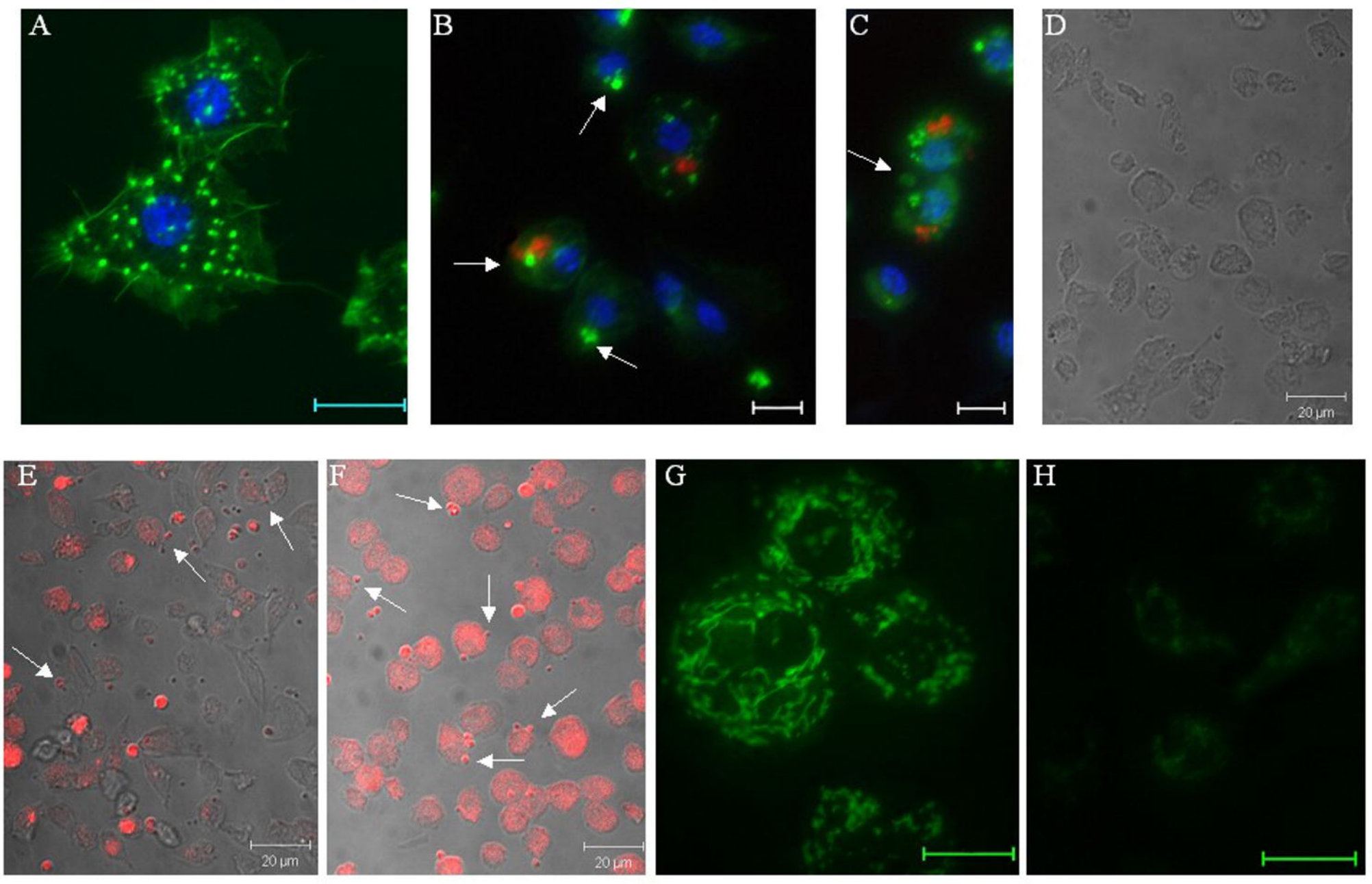
FIGURE LEGENDS

Fig. 1. Fluorescence and confocal microscopy images showing induced apoptosis in *T. molitor* haemocytes following *Neb*-colloostatin injections. A-C: morphological, apoptotic and cytoskeletal changes in haemocytes 1 hour after saline (control; A) and 1 nmole of *Neb*-colloostatin injection (B-C). All haemocytes were stained with SR-VAD-FMK reagent for caspase activity detection (red color), with Oregon-Green-phalloidin for staining of the F-actin cytoskeleton (green color) and with Hoechst-33258 for DNA staining (blue color). Arrows show aggregation of F-actin, visible as highly staining foci (B) and membrane blebs (C). Haemocytes of insects injected with peptide were less able to form filopodia in comparison to the control cells. (D-F): caspase activity and membrane blebbing in haemocytes 1 hour after injection of saline (control; D) and *Neb*-colloostatin in a dose of 1 (E) and 10 nmole/insect (F). The cells were stained with SR-VAD-FMK reagent for detection of caspase activity (red color). Many apoptotic bodies are observed in (E) or (F). Arrows show membrane blebbing. G-H: mitochondrial membrane depolarization caused by the *Neb*-colloostatin injections. In the control haemocytes, active mitochondria (G) stained with MitoTracker Green FM exhibit bright green fluorescence. Injection of 1 nmole of *Neb*-colloostatin causes depolarization of mitochondrial membranes as visualized by the inhibition of the uptake of MitoTracker Green FM (H). Scale bars indicate 20 µm.

Fig. 2. Confocal microscopy images showing the *in vitro Neb*-colloostatin-induced apoptotic effects in *T. molitor* haemocytes. A-B: apoptotic changes in haemocytes 1 hour after incubation in saline (control; A) and after 1 nmole of *Neb*-colloostatin treatment. The control (A) and the *Neb*-colloostatin-treated haemocytes (B) were stained with SR-VAD-FMK reagent for detection of active caspases (red color). Arrows show membrane blebs (B). Scale bars indicate 20 µm.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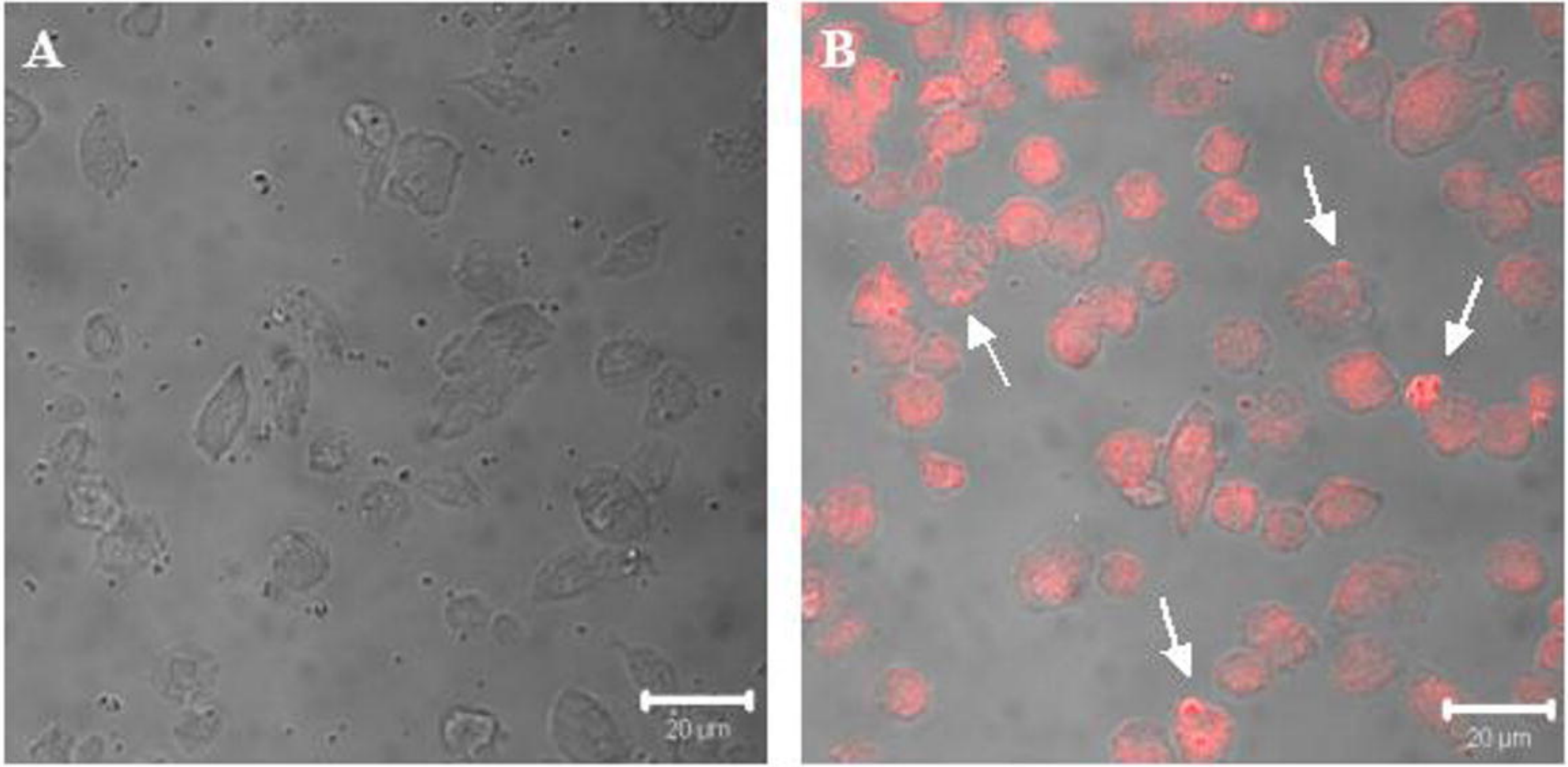
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